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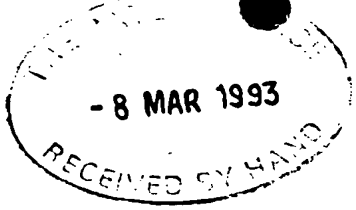

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Form 1/77

Patents Act 1977

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PROTEIN KINASES

This invention is related to that described in Application No. 9224057.1, filed 17th November 1992 (indicated below by [3]), and also that in the other Application filed on the same date as this and with the same title. In particular, this invention relates to nucleotides, peptides and antibodies raised thereto, and provides means whereby antibodies can be raised to peptides described in the two Applications identified above.

The following abbreviations are used herein: TGF-ß, transforming growth factor-ß; BMP, bone morphogenetic protein; PCR, polyermase chain reaction; ALK, activin receptor-like kinase; SDS, sodium dodecyl sulfate.

According to one aspect of the present invention, a novel

human cDNA clone for activin receptor-like kinase (ALK)-5 was obtained from a human erythroleukemia cell (HEL) cDNA library using polymerase chain reaction technology based on the amino acid sequence similarity to the mouse activin type II receptor and the *C. elegans daf-1* gene product. ALK-5 encodes a novel protein of 503 amino acids comprising a hydrophobic leader sequence, a cysteine-rich extracellular domain, a single transmembrane domain, and a cytoplasmic, putative serine/threonine kinase domain. The overall structure of ALK-5 is very similar to the activin and transforming growth factor-β (TGF-β) type II receptors and Daf-1. Analysis of mRNA expression in various cell lines and tissues showed that the ALK-5 gene is widely expressed as a major transcript of 5.5 kb, most abundantly in human placenta. Antibodies raised against a peptide corresponding to the intracellular juxtamembrane part of ALK-5, immunoprecipitated a 53 kDa protein from the cells transfected with the ALK-5 cDNA. After deglycosylation by endoglycosidase F treatment of the immunoprecipitated protein, the size shifted to 51 kDa. ALK-5 did not bind TGF-β1 and activin A; because of the similarity in structure with the activin and TGF-β type II receptors, it is possible that ALK-5 is a receptor for other member(s) of the TGF-β superfamily.

INTRODUCTION

Protein kinases play key roles in the signal transduction of various cellular activities (1). They can be classified into two categories by the target amino acids of their substrates, i.e. protein tyrosine kinases and protein serine/threonine kinases. Many protein tyrosine kinases have been identified as receptors for certain growth factors and as products of the oncogenes of transforming retroviruses. Binding of the ligands to protein tyrosine kinase recepors often induces dimerization of the receptors, which activates their cytoplasmic catalytic domains (reviewed in Ref. 2). The number of known receptor protein tyrosine kinases continues to grow; however, ligands for some of these receptors are yet unidentified. Protein serine/threonine kinases are also known to be important for the regulation of the cellular metabolism. In contrast to the protein tyrosine kinases, most of the protein serine/threonine kinases have been found as cytoplasmic proteins.

Transforming growth factor-β (TGF-β)[1] is a family of multifunctional proteins that regulate the growth, differentiation and metabolism of many different types of cells (for review, see Refs. 3, 4). TGF-β was first identified as a growth factor for normal rat fibroblasts in soft agar culture, but it is now known as a potent growth inhibitor for most cell types. TGF-β belongs to a large family of structurally related proteins, which includes three different mammalian isoforms of TGF-β (TGF-β1, -β2, and -β3), activins and inhibins, bone morphogenetic proteins (BMPs), and Müllerian inhibitory substance. The proteins of the TGF-β superfamily have a wide variety of biological activities and play important roles in the morphogenesis, e.g. during different stages of development.

TGF-β binds to three different types of receptors, i.e. type I, II, and III, in most cells (5). cDNAs for the TGF-β type III receptor (6-8) and the type II receptor (9) have been cloned; however, the structure of the type I receptor is not yet determined. The type III receptor is a membrane proteoglycan without any cytoplasmic protein kinase domain and may not be directly involved in the signal transduction. The TGF-β type II receptor (70 kDa) and the type I receptor (53 kDa), both of which are important for the signal transduction, may form a heteromeric complex; the type II receptor is needed for the binding of TGF-β to the type I receptor, and the type I receptor is required for the signal transduction induced by the type II receptor (10). For activin A and BMP-4, two types of affinity cross-linked complexes of about 60 kDa and 80 kDa have been identified on responsive cells and were denoted type I and type II receptors, respectively (11-13).

Among the type II receptors for the TGF-β superfamily proteins, the cDNA for the activin type II receptor was the first to be cloned (12). It was shown to have a putative protein serine/threonine kinase domain. A transmembrane serine/threonine kinase had previously been found in the Daf-1 protein (14), which is involved in the dauer larva development. Thereafter, another form of the activin type II receptor (activin type IIB receptor) (15, 16) and the TGF-β type II receptor (9) were cloned, both of which have putative serine/threonine kinase domains. These results suggest that receptor serine/threonine kinases form a new receptor family, which may include the type II receptors for other proteins in the TGF-β superfamily. Given the fact that about twenty proteins belong to the TGF-β superfamily, and that these proteins have prominent roles in cellular growth and metabolism, it is important to isolate new members in the activin/TGF-β type II receptor family. In order to achieve this goal, a polymerase chain reaction (PCR)-based approach, using cDNA from a human erythroleukemia (HEL) cell line, was developed. An advantage with this cell

line is that it responds to TGF-β and activin (11, 17), and would thus be expected to express activin and TGF-β type II receptors; these receptors could thus be used as controls to ascertain that the PCR was performed under appropriate conditions. Here, we report the isolation and characterization of a human cDNA clone encoding a novel receptor protein serine/threonine kinase denoted activin receptor-like kinase (ALK)-5.

MATERIALS AND METHODS

Preparation of mRNA and construction of a cDNA library - For construction of a cDNA library, poly(A)$^+$RNA was isolated from a human erythroleukemia cell line, HEL (American Type Culture Collection), by the guanidium isothiocyanate method (18), followed by use of a polyAT tract mRNA isolation kit (Promega). The isolated mRNA was used for the synthesis of random primed (Amersham) cDNA. A λgt10 cDNA library with $1x10^5$ independent cDNA clones was prepared by RiboClone cDNA synthesis system (Promega) and *in vitro* packaging kit (Amersham).

Generation of a cDNA probe by PCR - For the generation of cDNA probes by PCR (19), degenerated PCR primers were constructed based upon the amino acid sequence similarity between the mouse activin type II receptor (12) and Daf-1 (14) in the kinase domains II and VIII (nomenclature according to Ref. 1). Oligonucleotides were synthesized using Gene assembler plus (Pharmacia-LKB). The sense primer, B3-S, was a 25-mer oligonucleotide (5'-GCGGATCCGT(C/G/T)GC(A/C/T)GT(C/G/T)AA(A/G)AT(A/C/T)TT) derived from the conserved motif (VAVKIF in single letter code) in subdomain II with a 5' *Bam*HI restriction enzyme site. The antisense primer, EA-8S, was a 20-mer oligonucleotide (5'-CGGAATTC(A/G/T)GG(A/G/T)GCCAT(A/G)TA) derived

from the conserved sequence (YMAPE) in subdomain VIII with a 5' *Eco*RI site. PCR was performed in a 100 µl volume using first strand cDNA prepared from HEL mRNA, Taq polymerase (Perkin Elmer Cetus) and the oligonucleotide primers. The following program was used for PCR amplification; first 5 thermal cycles, each composed of 94°C (1 min), 50°C (1 min), 55°C (2 min), 72°C (1 min) followed by 20 thermal cycles of 94°C (1 min), 55°C (0.5 min), 72°C (1 min). A second round of PCR was performed with 3 µl of the first reaction as a template. After 25 thermal cycles, each composed of 94°C (1 min), 55°C (0.5 min), 72°C (1 min), the products with the expected sizes (~460 bp) were purified by agarose gel electophoresis. The PCR products were ligated into pUC19 (20) at *Bam*HI/*Eco*RI sites, and nucleotide sequences were determined by the di-deoxy chain termination method (21) using T7 DNA polymerase (Pharmacia-LKB). One of the PCR recombinants denoted 11.1 showed a novel sequence and was used for isolation of a full-length cDNA.

Isolation and characterization of a cDNA clone - The HEL cell cDNA library was screened with the insert of PCR recombinant 11.1 labeled by the Megaprime DNA labeling system (Amersham). Hybridization to nitrocellulose filters (Hybond-C extra, Amersham) was performed in 50% formamide, 5 x SSC (1 x SSC is 15 mM sodium citrate and 150 mM sodium chloride), 50 mM sodium phosphate, pH 6.9, 5 x Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS) and 0.1 mg/ml salmon sperm DNA at 37°C overnight. The filters were washed in 0.5 x SSC, 0.1% SDS at 55°C three times for 15 min, dried and exposed to Fuji X-ray films. Purification of a positive bacteriophage plaque was performed as described by Sambrook et al. (22). A clone denoted EMBLA was identified and subcloned into pBluescript SK (Stratagene) and thereafter sequenced on both strands. Compressions of nucleotides were resolved using 7-deaza-GTP (United States Biochemical Corp.).

Northern blot hybridization - Total RNA was prepared from HEL cells, a breast cancer cell line MCF-7, a lung carcinoma cell line A549, prostate carcinoma cell lines PC-3, PC-3U, and LNCaP, using the LiCl-urea precipitation method (23). MCF-7, A-549, PC-3, and LNCaP were obtained from American Type Culture Collection, and the PC-3U cells from Dr. Sten Nilsson (Department of Oncology, University Hospital, Uppsala, Sweden). For Northern blotting, 10 μg of total RNA was denatured at 65°C for 15 min in the presence of formaldehyde and formamide, electrophoresed in a 1% agarose gel containing formaldehyde and blotted to a Hybond-C extra filter. The filter was baked at 80°C under vacuum for 2 h, and then hybridized with a ^{32}P-labeled probe at 42°C overnight in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 0.1% SDS, 50 mM sodium phosphate, pH 6.9, and 0.1 mg/ml salmon sperm DNA. An *Eco*R1 fragment of 980 bp of the full-length cDNA clone, corresponding to the C-terminal part of the kinase domain and 3' untranslated region (nucleotides 1259-2232 in Fig. 1B) was radiolabeled by the Megaprime DNA labeling system, and used as a probe. The filter was washed two times in 0.5 x SSC, 0.1% SDS at 55°C for 15 min. A Northern blot filter with mRNAs from different human tissues was obtained from Clontech, hybridized with the same probe and washed as described above.

Transient expression of the ALK-5 cDNA - COS-1 cells obtained from American Type Culture Collection were used for transient expression. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics in 5% CO_2 atmosphere at 37°C. An ALK-5 cDNA (nucleotides (-76)-2232), which includes the whole coding region, was cloned into SV40-based expression vector pSV7d (24), and transfected into COS-1 cells by the calcium phosphate precipitation method (25). Briefly, cells were seeded into 6-well cell culture plates at a density of 5 x 10^5 cells/well, and transfected the following day with 10 μg of plasmid. After overnight

incubation, cells were washed three times with a buffer containing 25 mM Tris-HCl, pH 7.4, 138 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.6 mM Na_2HPO_4, and then incubated with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Transfected cells were used for metabolic labeling and immunoprecipitation, or for binding and affinity crosslinking, 2 days after transfection.

Antibodies for ALK-5 - A rabbit antiserum against ALK-5 denoted VPN was made against a synthetic peptide corresponding to amino acid residues 158-179. The peptide was synthesized with an Applied Biosystems 430A Peptide Synthesizer using t-butoxycarbonyl chemistry and purified by reversed phase high performance liquid chromatography. The peptide was coupled to keyhole limpet hemocyanin (Calbiochem-Behring) using glutaraldehyde, as described by Gullick et al. (26). The coupled peptide was mixed with Freund's adjuvant and used to immunize rabbits.

Metabolic labeling, immunoprecipitation and SDS-gel electrophoresis - Metabolic labeling of COS-1 cells was performed for 6 h in methionine- and cysteine-free MCDB 104 medium with 150 µCi/ml of [^{35}S]methionine and [^{35}S]cysteine (*In vivo* labeling mix, Amersham). After labeling, the cells were washed with 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, and then solubilized with a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1.5% Trasylol (Bayer) and 1 mM phenylmethylsulfonyl fluoride (Sigma). After 15 min on ice, the cell lysates were pelleted by centrifugation, and the supernatants were cleared one time with preimmune serum. Samples (1 ml) were then incubated with either 7 µl of preimmune serum or the specific antiserum VPN for 1.5 h at 4°C. For blocking, 10 µg of peptide was added together with the antiserum. Immune complexes were then given 50 µl of a protein A-Sepharose (Pharmacia-LKB) slurry (50%

packed beads in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.2% Triton X-10(and incubated for 45 min at 4°C. After incubation, the beads were spun down and washed four times with 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.2% SDS, followed by one wash in distilled water. The immune complexes were eluted by boiling for 5 min in the SDS sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) in the presence of 10 mM dithiothreitol, and analyzed by SDS-gel electrophoresis using 7-15% polyacrylamide gels (27). Gels were fixed, incubated with Amplify (Amersham) for 20 min, and subjected to fluorography.

Digestion with endoglycosidase F - Samples immunoprecipitaded by the VPN antiserum from metabolically labeled COS-1 cells transfected with the ALK-5 cDNA, were incubated with 0.5 mU of endoglycosidase F (Boehringer Mannheim Biochemica) in a buffer containing 100 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% Triton X-100, 0.1% SDS and 1% β-mercaptoethanol at 37°C for 24 h. Samples were eluted by boiling for 5 min in the SDS-sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorograpy.

Iodination of ligands, binding and affinity crosslinking - As ligands for the binding and crosslinking experiments were used recombinant human TGF-β1 and activin A, obtained from Dr. Arlen Thomason (Amgen Corp.) and Dr. Yuzuru Eto (Ajinomoto Co., Inc.), respectively. The ligands were iodinated using the chloramine T method according to Frolik et al. (28). Crosslinking experiments were performed as previously described (29). Briefly, COS-1 cells were transfected with pSV7d vector containing either the ALK-5 cDNA, the TGF-β type II receptor cDNA (obtained from a human foreskin fibroblast cDNA library)[2], or the mouse activin type II receptor cDNA (provided by Dr.

W.W. Vale). After two days, the cells were washed with binding buffer (phosphate-buffered saline containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$ and 1 mg/ml bovine serum albumin), and incubated on ice in the same buffer with radioiodinated ligands in the presence or absence of excess unlabeled ligands for 3 h. Cells were washed and crosslinking was done in the binding buffer without bovine serum albumin together with 0.14 mM of disuccinimidyl suberate (Pierce Chemical Co.) for 15 min on ice. The cells were harvested by the addition of 1 ml of detachment buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 0.3 mM phenylmethylsulfonyl fluoride). The cells were pelleted by centrifugation, then resuspended in 50 µl of solubilization buffer (125 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.3 mM phenylmethysulfonyl fluoride, 1% Trasylol) and incubated for 40 min on ice. Another centrifugation was done and supernatants were subjected to analysis by SDS-gel electrophoresis, followed by autoradiography.

RESULTS

Cloning and analysis of the ALK-5 cDNA

In order to identify novel receptor serine/threonine kinases that may bind to member(s) of the TGF-β superfamily, a PCR based approach was used with the prediction that these receptors share sequence similarities to each other. PCR was performed using HEL cell cDNA and degenerated primers that were based on conserved regions in the mouse activin type II receptor (12) and Daf-1 (14). One of the primers, B3-S, was derived from subdomain II, which includes the lysine residue predicted to be involved in ATP binding. The E8-AS primer was designed from the sequence in subdomain VIII, which is suggested to determine the specificity of serine/threonine kinases (1). Several different PCR products were obtained, including the human TGF-β type II

receptor and the human activin type II receptor. One PCR recombinant, 11.1 encoded a novel amino acid sequence (not shown), and was therefore used to screen a random primed HEL cell λgt10 cDNA library. Screening of about 1 x 10^5 independent clones yielded one positive clone, denoted EMBLA, that was further characterized. EMBLA has an insert of 5.3 kb with two internal *Eco*RI sites (Fig. 1A). Nucleotide sequencing revealed an open reading frame of 1509 bp, coding for 503 amino acids. The open reading frame is flanked by a 5' untranslated sequence of 76 bp, and a 3' untranslated sequence of 3.7 kb which was not completely sequenced. The nucleotide and deduced amino acid sequences of ALK-5 are shown in Fig. 1B. Noteworthy, the ALK-5 amino acid sequence has a 90 % sequence identity, but is not identical to the 11.1 PCR recombinant that was used as a probe for screening of the library.

Structure of the ALK-5 protein

In the 5' part of the open reading frame, only one ATG codon was found, which fulfilled the rules for initiation of translation (30). An in-frame stop codon was observed at nucleotides (-54)-(-52) in the 5' untranslated region. The predicted starting ATG codon is followed by a stretch of hydrophobic amino acid residues which has the characteristics of a cleavable signal sequence; therefore, the first ATG codon is likely to be used as a translation initiation site. A preferred cleavage site for the signal peptidase, according to von Heijne (31), is located between amino acid residues 24 and 25. The calculated molecular weight of the primary translated product of the human ALK-5 without signal sequence is 53,646. Another hydrophobic region, which represents a putative transmembrane domain (32) was found at amino acid residues 126-147. The putative transmembrane domain was followed by basic residues at His-149 and Arg-151, as is common for the cytoplasmic side of transmembrane regions (33). The extracellular domain has a cysteine-rich region with one potential N-glycosylation site.

The extracellular domain of ALK-5 shares little sequence similarity (less than 20% identity) with the other activin/TGF-β type II receptor family proteins; however, a fair alignment of the cysteine residues in these proteins could be performed (Fig. 2). The cytoplasmic part has a putative protein kinase domain. A consensus sequence for the binding of ATP (Gly-X-Gly-X-X-Gly, followed by Lys further downstream in subdomain II) was found in ALK-5 at amino acids 212 to 217 and 232, respectively. The putative kinase domain of ALK-5 contains most of the consensus motifs specific for serine/threonine kinases (Fig. 2, see Discussion), and shows a 42% sequence identity to the mouse activin type II receptor, 41% to the mouse activin type IIB receptor, 42% to the human TGF-β type II receptor, and 43% to Daf-1.

Expression of ALK-5 mRNA in different cells and tissues

The ALK-5 mRNA size and distribution were determined by Northern blot analysis. Fig. 3A shows that a 5.5 kb ALK-5 mRNA is expressed in all cell lines tested, except for LNCaP, a human prostate cancer cell line. A minor transcript of 3.4 kb was also observed in most of the cells. mRNA for ALK-5 is also ubiquitously expressed in different human tissues (Fig. 3B). The transcript of 5.5 kb was most abundant in placenta and least in brain and heart. The 3.4 kb transcript was not observed in the tissues. The size of the major transcript (5.5 kb) suggests that the obtained cDNA clone was close to full length.

Transient expression of ALK-5 in COS-1 cells

ALK-5 cDNA was subcloned into the SV40-based expression vector pSV7d and transfected into COS-1 cells. Two days after transfection, the cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. An antiserum was raised against a synthetic peptide corresponding to amino acids 158-179 which is the juxtamembrane part of the cytoplasmic domain, and used for

immunoprecipitation of metabolically labeled COS-1 cells transfected wit ALK-5 cDNA. One component of 53 kDa was seen when the samples were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). This component was not seen when 10 μg of peptide was added together with the antiserum. Moreover, the preimmune serum did not recognize the 53 kDa component, and it was not found in samples derived from untransfected COS cells using either preimmune serum or the antiserum. We conclude that the 53 kDa component represents the ALK-5 protein. When the immunoprecipitated sample was treated with endoglycosidase F, that hydrolyzes the complex- and high mannose-types of N-linked carbohydrates, the 53 kDa band shifted to 51 kDa. The extracellular domain of ALK-5 contains one potential acceptor site for N-glycosylation and the size of the deglycosylated protein is close to the predicted size of the core protein. A 21 kDa component also appeared in the endoglycosidase F-treated samples; the relation of this molecule to ALK-5 remains to be determined.

TGF-β1 and activin A do not bind to ALK-5

In order to investigate whether ALK-5 binds TGF-β1 or activin A, COS-1 cells were transfected with a pSV7d vector containing ALK-5 cDNA. As positive controls, cells were transfected with the expression vectors containing the TGF-β type II receptor or activin type II receptor cDNAs. Neither ^{125}I-TGF-β1 nor ^{125}I-activin A bound at higher levels to the ALK-5 transfected cells, compared to the untransfected cells (data not shown). In contrast, ^{125}I-TGF-β1 and ^{125}I-activin A bound to the cells transfected with the cDNAs for the corresponding receptors, and components of 90-100 kDa were observed upon analysis by SDS-gel electrophoresis and autoradiography after crosslinking (data not shown).

DISCUSSION

Protein kinases have conserved catalytic domains of about 250-300 amino acid residues, that are responsible for the enzymatic activity (1). Using short stretches of highly conserved amino acid sequences and PCR technology, many new receptor protein tyrosine kinases have been obtained (34-37). We have extended this strategy to the cloning of receptor serine/threonine kinases of the activin/TGF-β type II receptor family. Among several different PCR primers, combination of those designed from the amino acid sequences of kinase domains II and VIII allowed us to obtain several PCR recombinants encoding novel amino acid sequences. The predicted amino acid sequences of the PCR recombinants obtained by these primers showed conservation of specific amino acid sequences found in the activin/TGF-β type II receptor family.

ALK-5, obtained using the PCR recombinant 11.1, has a 98 amino acid extracellular domain with a cysteine-rich region, a single transmembrane domain, and a 356 amino acid intracellular domain with a putative serine/threonine kinase region (Fig. 1). Cysteine-rich regions have also been found in the activin/TGF-β type II receptor family, and the positions of the cysteine residues are similar to those in ALK-5 (Fig. 2).

The subdomains VI and VIII of protein kinases contain amino acid sequences that are useful to predict the specificity regarding which amino acids are phosphorylated (1). In ALK-5, the sequence in subdomain VI is HRDLKSKN, which is very similar to the activin/TGF-β type II receptors. It is also similar to other cytoplasmic serine/threonine kinases, but is very different from receptor protein tyrosine kinases. Similarly, the sequence in subdomain VIII contains GTKRYMAPE, which fits very well to the consensus sequence of

serine/threonine kinases. These results suggest that ALK-5 has a protein kinase specific for serine and threonine residues. However, recent reports suggested that some putative serine/threonine kinases, e.g. mitogen-activated protein kinases, Clk/Sty kinase and Wee1 kinase, are dual specificity kinases, which phosphorylate serine, threonine as well as tyrosine residues (38). It was also shown that purified activin type IIB receptor phosphorylates serine, threonine and tyrosine residues (39). Whether ALK-5 also has a kinase with dual specificity, remains to be elucidated.

Similar to the other activin/TGF-β type II receptor family proteins, the protein kinase domain of ALK-5 is interrupted by short streches of amino acids between subdomain VIA and VIB and between subdomain X and XI (Fig. 1 and 2). Based on the sequence similarity with the other activin/TGF-β type II receptors, the C-terminus of the kinase domain of ALK-5 was set at Gln-498, and thus the C-terminal tail of ALK-5 has only 5 amino acids, which is considerably shorter than the other activin/TGF-β type II receptors (23-28 amino acids). In the receptor protein tyrosine kinases, kinase inserts and C-terminal tails contain tyrosine residues that are autophosphorylated after ligand binding, and constitute regions which associate with down-stream components in the signal transduction pathway (2). Tyrosine residues are not found in these portions of ALK-5, whereas some serine and threonine residues are found in the kinase inserts; whether they are autophosphorylation sites remains to be determined. The juxtamembrane part of the cytoplasmic domain of ALK-5 has a low amino acid sequence similarity to the other activin/TGF-β type II receptors (11-23% amino acid identity) (Fig. 2) and other ALKs (36-51% identity, see below), and contains some serine, threonine as well as tyrosine residues.

ALK-5 was ubiquitously expressed in different cells and tissues, and most strongly expressed in human placenta. Using different PCR recombinants, we have obtained four other receptor serine/threonine kinases (ALK-1 to -4; the ALK-4 cDNA is truncated in the extracellular domain)[3]. In the kinase domain, ALK-5 is more similar to the other ALKs (63-90% amino acid sequence identity) than to the other activin/TGF-β type II receptors (41-43% identity), whereas the sequence identity in the extracellular cysteine-rich domain is below 20 % for ALK-1 to -3 as well as the activin/TGF-β type II receptors. Some of the ALK family proteins are ubiquitously expressed, but analyses of the tissue distribution of their mRNA showed different patterns of expression[3], indicating different roles of each protein *in vivo*.

Transient expression of the ALK-5 cDNA, followed by affinity crosslinking of ^{125}I-TGF-β1 and ^{125}I-activin A, did not show any binding of these ligands to ALK-5. Because of the conservation of the cysteine residues in the putative ligand-binding domains between ALK-5 and the activin/TGF-β type II receptors, ALK-5 may also bind the protein(s) of the TGF-β superfamily. In addition, the fact that ALK-5 has a molecular mass of 53 kDa, leaves the possibility open that ALK-5 is a type I receptor for member(s) of the TGF-β superfamily. Given that these ligands act in autocrine and/or paracrine fashions, expression profiles of the ligands and receptors may correlate to each other. Therefore, more detailed information about the expression of ALK-5 in different tissues and comparison with those of the TGF-β superfamily proteins, will be valuable to predict the identity of the ligand(s). Such studies are under way in our laboratory.

Acknowledgments - We thank A. Thomason, Y. Eto, and W.W. Vale fo providing recombinant human TGF-β1, activin A, and cDNA for the mouse activin type II receptor, respectively. We are aslo grateful to Ulla Engström and Christer Wernstedt for preparing the synthetic peptide and oligonucleotides, respectively. We thank Jan Saras for valuable help and discussion. Peter ten Dijke is supported by an EMBO postdoctoral fellowship.

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FIGURE LEGENDS

Fig. 1. cDNA cloning and sequencing of ALK-5. The structure of the cDNA clone EMBLA is schematically illustrated in (A). Boxes represent the coding region; filled box indicates the signal peptide, shaded box the transmembrane domain, and hatched box the intracellular kinase domain. The *Eco*RI cleavage sites are also indicated. The nucleotide and deduced amino acid sequences of EMBLA are shown in (B). Nucleotides and deduced amino acids are numbered to the right, starting with the proposed initiating methionine codon. The N-terminal hydrophobic signal sequence and transmembrane domain are overlined (thin lines), and the potential N-glycosylation site is underlined (thick line). Cysteine residues in the extracellular domain are boxed and the stop codon which ends the open reading frame is marked with an asterisk. The ends of the kinase domain are indicated by arrows. Indicated by half arrows are the regions in the kinase domain that were used for construction of the primers for PCR. The in-frame stop codon found in the 5' untranslated region is underlined with a thin line.

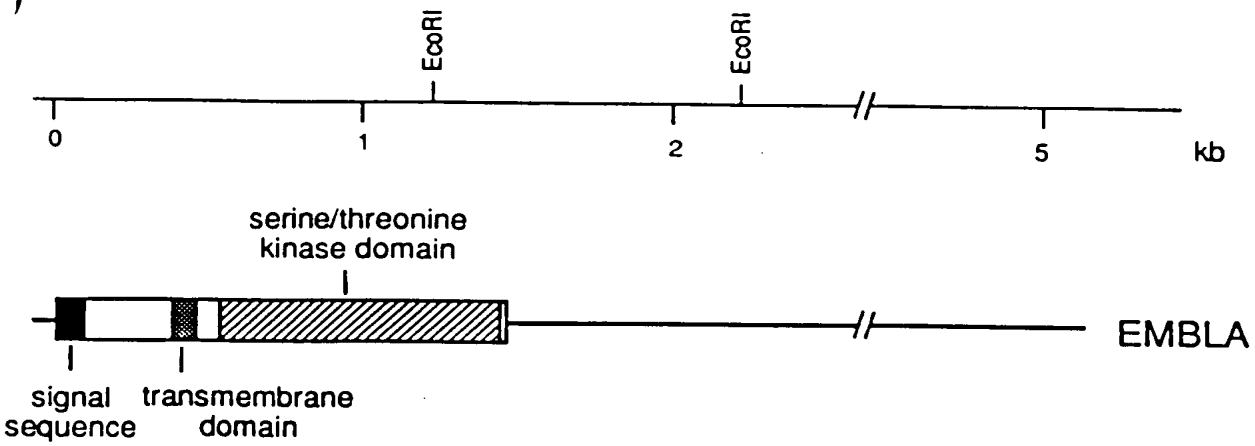
Fig. 2. Comparison of ALK-5 with activin and TGF-β type II receptors. Amino acid sequence of the human ALK-5 was compared with those of the human activin type II receptor (ActR-II) (40, 41), and the human TGF-β type II receptor (TGFβR-II) (9). Alignment was perfomed with the Clustal computer alignment program (42), with some manual adjustment. Identical amino acids are boxed and cysteine residues in the extracellular domain are shaded. The hydrophobic leader sequence and transmembrane domain of ALK-5 are overlined. The ends of the kinase domain and the kinase inserts are indicated by arrows and underlined by thick lines, respectively. The kinase subdomains are shown with roman numerals according to Hanks et al. (1).

Fig. 3. Northern blot analysis of ALK-5. (A) Expression of ALK-5 mRNA was investigated in different human cell lines. RNA was prepared from various cell lines and 10 µg of total RNA was loaded per lane, electrophoresed in a 1% agarose gel containing formaldehyde and blotted to a Hybond C extra membrane. The filter was probed with a radiolabeled 980 bp internal *Eco*RI fragment. HEL, a human erythroleukemia cell line; PC-3, PC-3U and LNCaP, human prostate carcinoma cell lines; A549, a human lung carcinoma cell line; MCF-7, a human breast carcinoma cell line. In the ethidium bromide staining of the gel the ribosomal RNA showed equal staining in all lanes. (B) Expression of ALK-5 in different human tissues. A multiple human tissue blot (Clontech) was hybridized with a radiolabeled 980 bp internal *Eco*RI fragment. Each lane contained 2 µg of polyadenylated RNA from the indicated tissues.

Fig. 4. Transient expression of ALK-5 and immunoprecipitation by ALK-5 antibody. Control COS-1 cells and COS-1 cells transfected with the pSV7d expression vector containing the ALK-5 cDNA were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 6 h. The cell lysates were then subjected to precipitation using preimmune serum (pre) or VPN antiserum (immune). Blocking was performed with 10 µg of peptide together with the VPN antiserum (block). Enzymatic deglycosylation of ALK-5 was performed with 0.5 mU of endoglycosidase F (endo F) at 37°C for 24 h. Specific bands are indicated by arrows. Size markers are shown on the left.

Fig 1

A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EcoRI
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kinase domain
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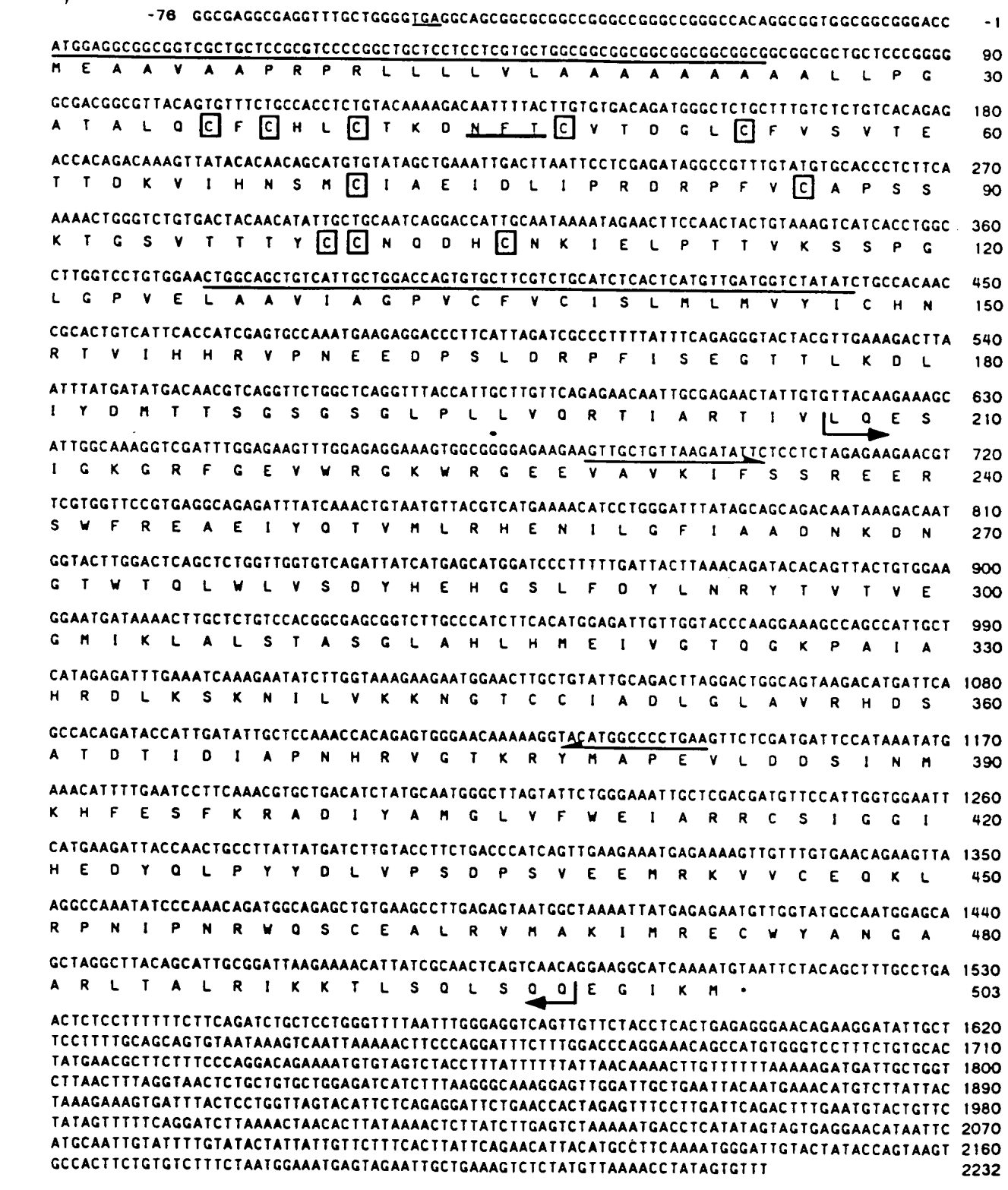
signal
sequence
transmembrane
domain

Fig. 1A

Fig 1

B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A T A L Q C F C H L C T K D N F T C V T D G L C F V S V T E 60
ACCACAGACAAAGTTATACACAACAGCATGTGTATAGCTGAAATTGACTTAATTCCTCGAGATAGGCCGTTTGTATGTGCACCCTCTTCA 270
T T D K V I H N S M C I A E I D L I P R D R P F V C A P S S 90
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L G P V E L A A V I A G P V C F V C I S L M L M V Y I C H N 150
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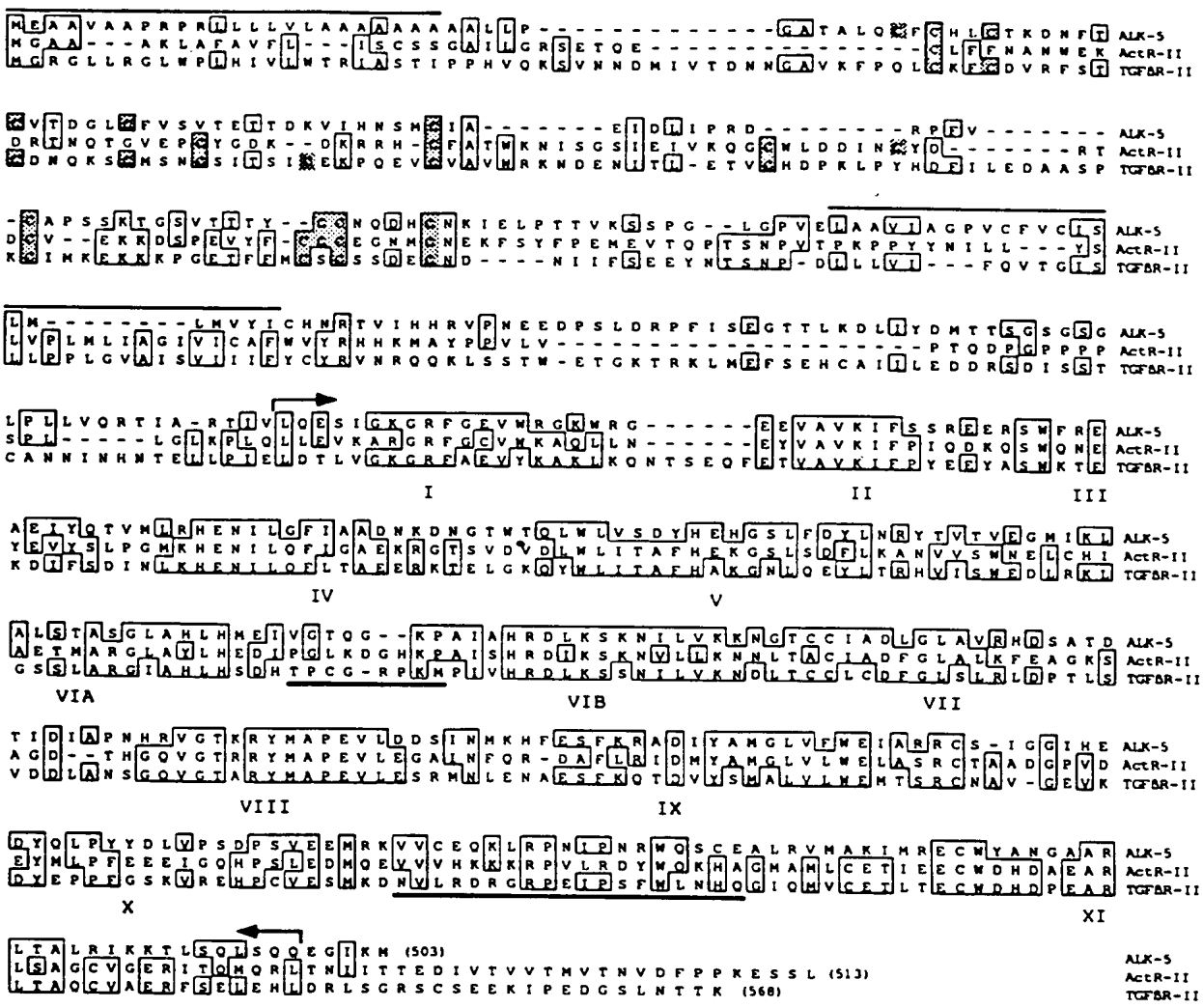
Fig. 1B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ActR-II
TGFβR-II
I
II
III
IV
V
VIA
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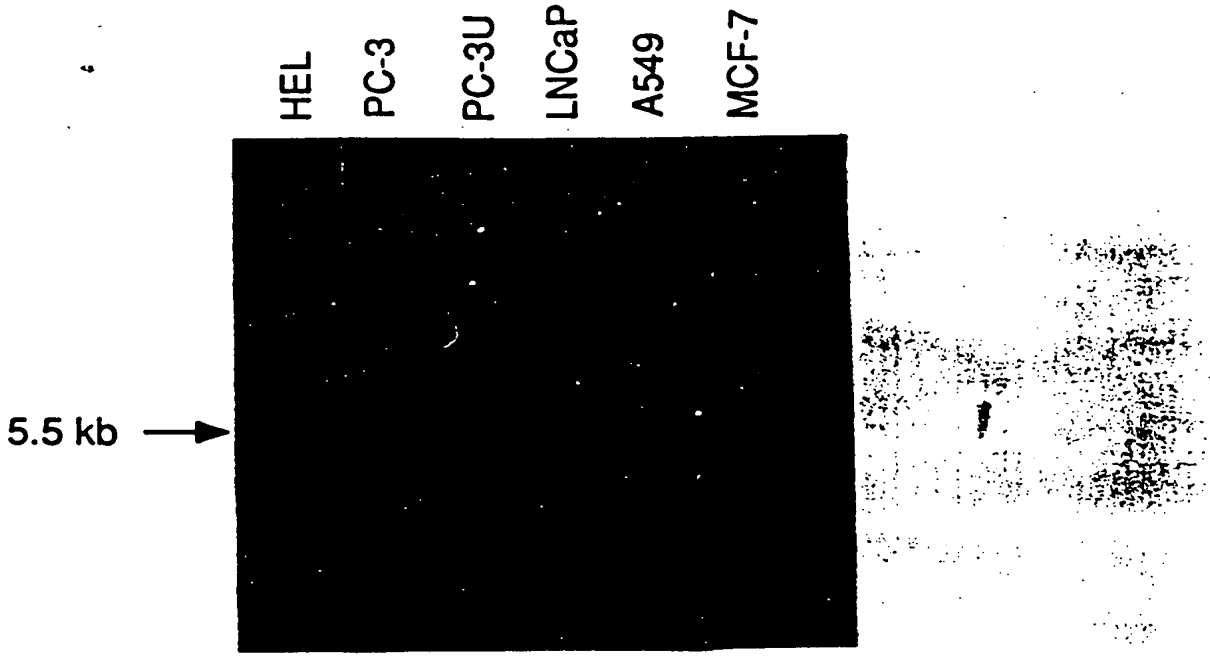
VII
VIII
IX
X
XI
(503)
(513)
(568)

Fig. 2

Fig. 3

A)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B)

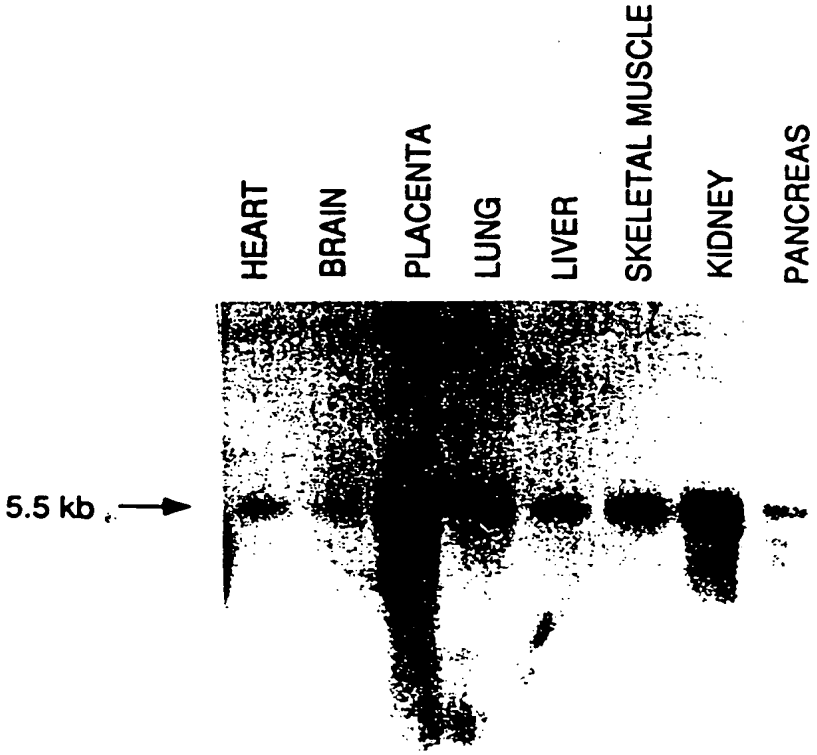
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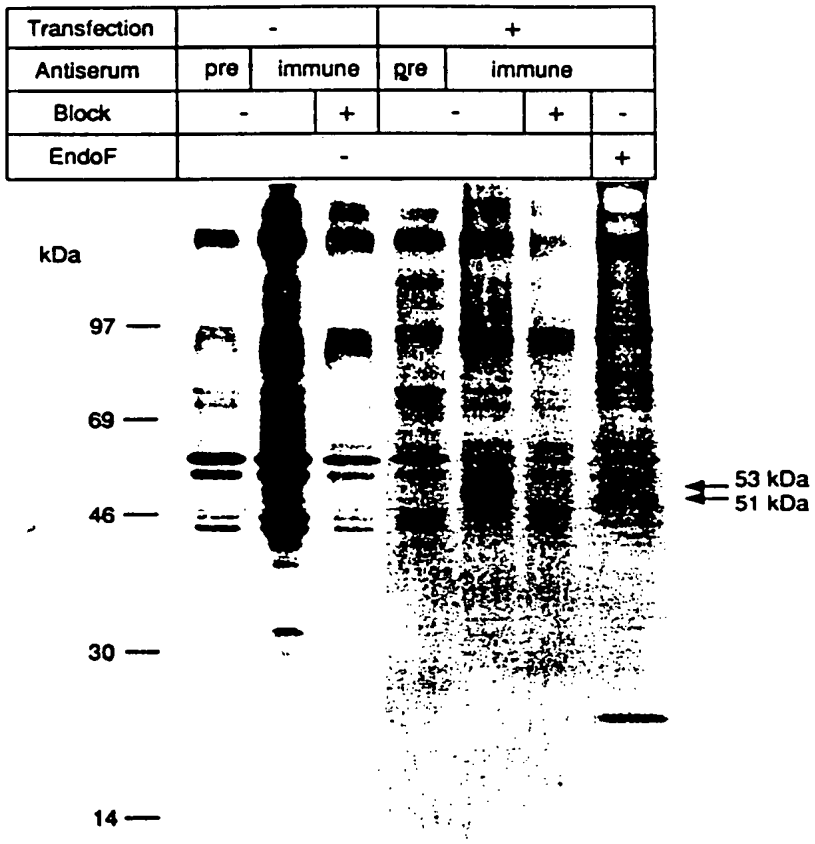
Fig. 4


Transfection
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Antiserum
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Block
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EndoF
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+
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